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# SHORT PAPER

# *Pseudomonas aeruginosa* as a cause of infectious diarrhoea

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## SUMMARY

*Pseudomonas aeruginosa* is not generally considered a cause of infectious diarrhoea. However, it was the predominant organism isolated from the faeces of 23 unrelated, hospital outpatients investigated in the course of a year for persistent (> 1 week duration) diarrhoea. To investigate the possible aetiological role of *P. aeruginosa*, these patient histories were reviewed and a selection of their faecal isolates were investigated *in vitro* ($n \geqslant 10$) and *in vivo* ($n = 2$) for virulence. The patients had a mean age of 60 years, were receiving antibiotics and/or had an underlying illness. Extensive microbiological investigations identified no other potential or recognized enteropathogen in the faeces of 20 of these patients. More than 40% of the isolates tested were able to adhere to HEp-2 cells and exhibited twitching motility (type IV pili), properties indicative of their ability to colonize the human intestine. Cytotoxic activity was demonstrated in bacterium-free cell supernatants of over 80% of isolates; supernatants of four isolates tested in infant mice were weakly enterotoxigenic. Two isolates intragastrically inoculated into clindamycin pre-treated rats established persistent infections and induced signs and symptoms of enteritis. Overall these findings suggest that *P. aeruginosa* can cause diarrhoea particularly in immunodeficient individuals.

*Pseudomonas aeruginosa* is an environmental organism (water, soil and on plants). Although it is occasionally detected as part of the normal human microflora of healthy individuals, it can cause serious infections in immunocompromised and critically ill patients [1]. It has only rarely been implicated as a cause of infectious diarrhoea, with reported cases predominantly involving individuals suffering haematologic malignancies and neutropaenia secondary to chemotherapy [2–4] or epidemics in infants [5–8]. Most studies have demonstrated only the simultaneous presence of the organism and diarrhoea in the absence of other known pathogenic agents. Hence, the role of *P. aeruginosa* in the production of enteritis remains in doubt. In this report, we present evidence of a causative relationship between *P. aeruginosa* and diarrhoea, particularly in immunocompromised individuals.

In the course of a 1-year study, 23 patients presented to the Launceston General Hospital, Launceston, Tasmania, Australia with persistent (> 1 week duration) pseudomonas-associated diarrhoea. The case histories of these patients were reviewed (case notes and interviews). Based on these histories, *P. aeruginosa* isolates from selected patients (those with prolonged diarrhoea and minimal obvious pre-disposing risk factors) were subsequently examined for virulence properties and their ability to cause enteritis in rats.

Microbiological examination of diarrhoeal faeces included direct microscopy for ova, cysts, parasites, white and red blood cells and a cold acid-fast stain (Kinyoun stain) to detect *Cryptosporidium* oocysts [9]. Faeces were also tested for rotavirus (Serobact

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Table 1. *Virulence factors of* P. aeruginosa *isolates from diarrhoeal faeces*

| Virulence factor | No./total positive (%) |
|---|---|
| Adhesion (HEp-2 cells)* | 5/11 (45) |
| Twitching motility† | 4/10 (40) |
| Cytotoxic activity‡ | 9/11 (82) |
| Enterotoxic activity§ | 2/4 (50) |

* $\geqslant 5$ bacteria per HEp-2 cell [12].
† Zone size ($20 \pm 3$ mm) comparable with a type IV piliated *P. aeruginosa* control strain, PAO1 [11].
‡ $\geqslant 50\%$ Vero cells affected [13].
§ Suckling mouse intestinal weight to remaining body weight ratio $\geqslant 0{\cdot}075$ [10, 13].

Rotavirus Latex Slide Test, Medvet Science Pty. Ltd., Adelaide, Australia). They were cultured on *Campylobacter* and *Aeromonas* selective agars, MacConkey and Xylose Lysine Desoxycholate (XLD) agars, before and after enrichment in selenite F broth (48 h) to screen for recognized aerobic/facultative aerobic bacterial enteropathogens. Anaerobic culture on Clostridium Difficile Selective Agar containing Clostridium Difficile Selective Supplement SR6 (Oxoid) and testing of faeces for *C. difficile* toxin A (C. Difficile Toxin A Test, Oxoid) was also carried out.

All *P. aeruginosa* isolates were stored in minimum maintenance medium (room temperature) [10]. Isolates chosen for study were tested for virulence properties indicative of their capacity to induce diarrhoea (Table 1). Full details of these virulence assays are given elsewhere [10–13]. In brief, isolates ($1 \times 10^6$ c.f.u.) were examined for their ability to adhere to HEp-2 cells after growth (37 °C for 24 h) in tryptone soya broth supplemented with 6·0 g/l yeast extract L21 (Oxoid), TSBY. Each isolate was assessed on triplicate coverslip cultures of semi-confluent HEp-2 cells on two or more occasions. Strains with $\geqslant 5$ bacteria per HEp-2 cell were defined as 'adherent', those with $\geqslant 10$ bacteria per cell as 'highly adherent'. The ability of isolates to exhibit twitching motility was determined using an agar assay developed for *P. aeruginosa* [11]. A known type IV piliated *P. aeruginosa* control strain, PAO1 was included in each assay. This strain had a zone size of $20 \pm 3$ mm which was defined as positive for twitching motility. For toxin assays, bacteria were cultured in TSBY at 37 °C for 24 h with shaking (200 rpm, Paton Industries Orbital Shaker, Adelaide, Australia) and bacterium-free broth supernatants prepared. These were assessed for cytotoxic activity against Vero cells. A positive cytotoxin score was recorded if $\geqslant 50\%$ Vero cells were affected (rounding of cells and detachment from the monolayer) after 18–20 h exposure to the supernatant. Enterotoxic activity was determined by measuring fluid accumulation in the intestines of 2–3 day-old infant BALB/c mice (three or more animals per group) 3 h after intragastric inoculation with the supernatants (100 $\mu$l). The intestinal weight to remaining body weight ratio (IW:RBW) was determined and scores $\geqslant 0{\cdot}075$ were considered positive [10].

Two isolates, PA1 and PA2, (properties summarized in Table 2) were also tested for their ability to cause enteritis in rats using a protocol described by Harberberger and colleagues [14]. To enable recovery of these strains from rat faeces, they were first cultured in TSBY containing streptomycin and ampicillin to ensure they were resistant to these antibiotics. Rats were pre-treated with clindamycin prior to inoculation to reduce the normal anaerobic flora of the gut and hence facilitate bacterial colonization. In brief, male hooded Wistar rats ($\sim 150$ g) received a daily intramuscular injection of clindamycin (30 mg/kg) for 3 consecutive days prior to bacterial challenge. Bacteria ($\sim 10^{10}$ c.f.u. suspended in 0·5 ml sterile saline) were delivered intragastrically over the next 3 consecutive days. Isolate PA1 was tested once (4 rats); isolate PA2 on two occasions (8 rats). Control animals, included in each experiment, received saline injections instead of clindamycin pretreatment (2 rats per experiment), or clindamycin followed by saline (no bacterial challenge) (2 rats per experiment). Animals were examined daily for evidence of diarrhoea. Faecal pellets from individual animals were weighed and cultured for *P. aeruginosa* following blending (speed 6, 30 s bursts) with a known volume (2–6 ml) of sterile saline (Omnimixer, Omni International, CT, USA). *P. aeruginosa* was quantitated from serial dilutions of homogenized faeces by plate counts on tryptone soya yeast extract agar (TSAY) containing 100 $\mu$g/ml streptomycin and 25 $\mu$g/ml ampicillin (24 h, 37 °C). The organism was not recovered from animals prior to bacterial challenge. Animals from each group were sacrificed at intervals after the last bacterial challenge (Fig. 1). Tissues (1 cm sections) from defined points along the intestinal tract and including the duodenum, the jejunum, three points along the ileum, the caecum and colon, were taken for histological examination (haematoxylin-and-eosin staining).

The 23 patients reviewed comprised 11 males and 9 females; for 3 patients the sex was not recorded. They

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Table 2. P. aeruginosa *isolates tested in clindamycin-treated rats*

| | Isolate* | |
|---|---|---|
| | PA1 | PA2 |
| Patient source | | |
| Sex | Male | Male |
| Age | 28 years | 7 years |
| Other isolates from microbiological culture | None | None |
| Symptoms other than diarrhoea | Intermittent abdominal pain | None |
| Previous clinical history | None relevant | Rotavirus infection as neonate (4 days old)† |
| Virulence factors | | |
| Adhesion (bacteria/HEp-2 cell) | 2·4 ± 1·4 | 12·3 ± 4·2 |
| Twitching motility | − | + |
| Cytotoxic activity‡ | + | + |
| Enterotoxic activity§ | ± | ± |

* *P. aeruginosa* isolated in pure culture (enteric selective media); no other potential pathogens detected.
† Repeated bouts of persistent diarrhoea reported since infancy.
‡ ⩾ 50% Vero cells affected.
§ Supernatants tested in the suckling mouse assay gave borderline enterotoxin scores (0·073 and 0·072, respectively).

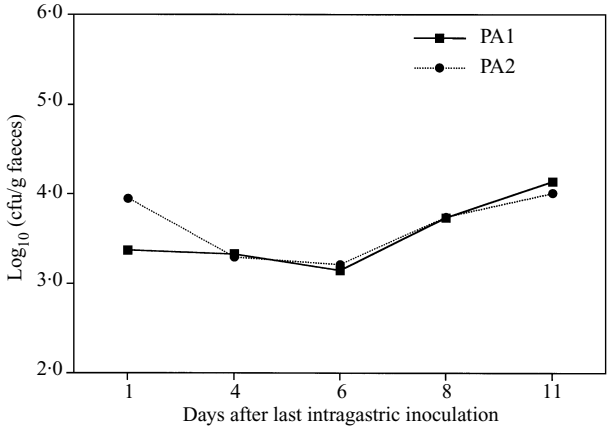


**Fig. 1.** Mean numbers of *P. aeruginosa* ($\log_{10}$ c.f.u./g of faeces) shed at different time intervals by groups of clindamycin-pretreated rats challenged with *P. aeruginosa* isolates, PA1 or PA2. Due to the periodic sacrifice of animals for histology, values at the different time points are from decreasing numbers of animals. On days 1, 4, 6, 8 and 11 the means for PA1 are from 4, 4, 3, 2 and 2 rats and for PA2 they are from 8, 7, 6, 5 and 5 rats, respectively.

had a mean age of 60 years (range 3–90 years). All reported recent, prolonged episodes of diarrhoea; several had a history of recurrent diarrhoeal episodes (months to years). *P. aeruginosa* was a consistent feature and was repeatedly isolated from faeces. In only three cases was another potential enteropathogenic organism isolated (an *Aeromonas* spp., a *Campylobacter* spp. and rotavirus, respectively). Frank blood and/or red cells were recorded in three cases and three patients reported vomiting in association with their diarrhoeal episodes. All but one of the individuals could be considered immunocompromised in some way either through age, underlying illness, past history or antimicrobial or other drug therapy. For 10 patients (mean age 81 years, range 74–90 years), who were well except for their diarrhoeal symptoms, age was the only risk factor. Seven patients (mean age 69, range 39–85 years) had underlying illnesses (including one each of hiatus hernia, gastritis, breast cancer and pancreatitis). Six of the latter, and one other patient, were receiving drug therapy for these conditions or antimicrobials for conditions unrelated to their diarrhoea. Another patient had an extensive history of recurrent bowel problems and three further patients had recognized or potential enteropathogens isolated from their faeces in addition to *P. aeruginosa*.

Table 1 shows the results of *in vitro* virulence assays on selected *P. aeruginosa* isolates. These clearly possessed the ability to adhere to and colonize the human intestine. Adhesion to HEp-2 cells and twitching motility (thought to be important in the translocation and colonization of *P. aeruginosa* at the mucosal surface [11]) was demonstrable in > 40% of isolates tested under the given assay conditions;

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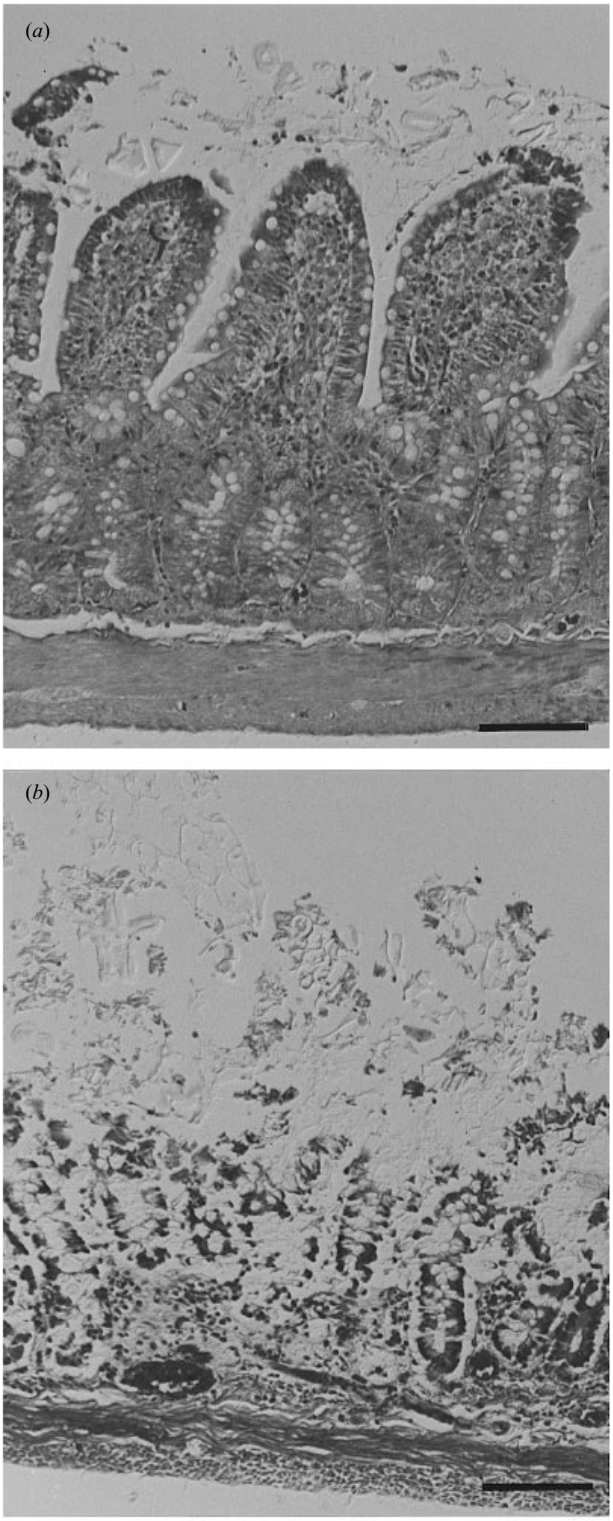


**Fig. 2.** Haematoxylin-and-eosin stained rat ileal tissue (11 days post challenge). (*a*) Tissue from control rat (saline injections only). (*b*) Tissue from clindamycin-pretreated rat inoculated with *P. aeruginosa* isolate PA2. Focal ulceration and necrosis with sloughing of portions of the villi were seen. Bar, 1 mm.

prolonged faecal shedding was also observed in inoculated rats (Fig. 1). A high proportion ($> 80\%$) of isolates produced detectable cytotoxic activity. Supernatants of 4 strains induced detectable intestinal fluid accumulation in suckling mice, although only 2 isolates produced an intestinal weight (IW) to remaining body weight (RBW) ratio of $\geqslant 0{\cdot}075$ (criterion for positivity).

Table 2 summarizes background patient data and virulence properties for the two isolates selected for *in vivo* pathogenicity investigations. Both were from younger patients with no obvious, recent predisposing risk factors. Both colonized clindamycin-treated rat intestines. Their shedding in faeces after bacterial challenge is shown in Figure 1. Pseudomonas numbers declined until 6 days post challenge, rose steadily thereafter and the organisms were still being shed from test animals at concentrations $> 10^4$ c.f.u./g faeces at the termination of the experiment (11 days). Clindamycin pretreatment was necessary to facilitate such long-term colonization as the numbers of *P. aeruginosa* continued to decline from 6 days (to $< 5 \times 10^2$ c.f.u./g faeces) in animals which received saline pretreatment only (Fig. 1). Pseudomonads were not
shed from unchallenged, clindamycin-treated, control rats. Unformed stools were occasionally seen in challenged animals (twice with isolate PA2 and once with isolate PA1), but not in control animals. Both strains caused damage (shortening and necrosis) to the villi tips throughout the small intestine. Changes were most severe in the ileum; the large intestine was essentially unaffected. From 6 days post challenge, ulceration and severe necrosis of ileal architecture occurred in over 75% of animals inoculated with either strain (Fig. 2). No such changes were seen in the ileal tissues of control animals.

This study has, therefore, confirmed that *P. aeruginosa* may occur as sole potential pathogen in patients with diarrhoea [2–8]. Diarrhoea-associated isolates were also shown to produce toxic activities that could precipitate diarrhoea. Other investigators have reported cytotoxic and invasive phenotypes of *P. aeruginosa* [15, 16]. Moreover, this study has shown that diarrhoea-associated *P. aeruginosa* isolates can induce signs and symptoms of enteritis in antibiotic-treated rats. These results suggest that *P. aeruginosa* can be an aetiological agent of diarrhoea particularly in immunodeficient or antibiotic-treated individuals. Such patients may well benefit from specific anti-microbial therapy to eliminate the organism from their intestines.

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